Ethanolic Crude Extract and Flavonoids Isolated from *Alternanthera maritima*: Neutrophil Chemiluminescence Inhibition and Free Radical Scavenging Activity

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Extracts from *Alternanthera maritima* are used in Brazilian folk medicine for the treatment of infectious and inflammatory diseases. Bioassay-guided fractionation of *A. maritima* aerial parts yielded an ethanolic crude extract, its butanolic fraction and seven isolated flavonoids (two aglycones, two *O*-glycosides and three *C*-glycosides) with antioxidative activity. The ability of these samples to scavenge enzymatically generated free radicals (luminol-horseradish peroxidase-H_2O_2 reaction) and inhibit reactive oxygen species (ROS) production by opsonized zymosan-stimulated human neutrophils (PMNLs) was evaluated by chemiluminescence methods. In both assays, the butanolic fraction was significantly more active than the ethanolic crude extract, the flavonoid aglycones had high inhibitory activities and the *C*-glycosylated flavonoids had no significant effect even at the highest concentration tested (50 μmol/L). However, the *O*-glycosylated flavonoids inhibitory effects on chemiluminescence were strongly dependent on the chemical structure and assay type (cellular or cell-free system). Under the conditions tested, active samples were not toxic to human PMNLs.

Key words: Alternanthera maritima, Flavonoids, Neutrophil

Introduction

Polymorphonuclear leukocytes (PMNLs) or neutrophil-derived reactive oxygen species (ROS) constitute one of the main body's defense mechanisms against microorganisms. However, PMNL excessive oxidative metabolism may cause unwanted tissue damage and the setting in of inflammatory reactions such as rheumatoid arthritis (Babior, 2000). The involvement of PMNL-derived ROS in the physiopathology of several inflammatory diseases has attracted interest in the search of new natural compounds able to modulate this process (Kabeya *et al*., 2002; Kanashiro *et al*., 2004).

Many plant species (Salvador *et al*., 2002; Cai *et al*., 2003; Lagrota *et al*., 1994) used in nutrition and Brazilian folk medicine for the treatment of several diseases such as infections, inflammation and fever (Macedo *et al*., 1999; De Souza *et al*., 1998; Gorinstein *et al*., 1991; Si-Man *et al*., 1988; Siqueira, 1987) are included in the Amaranthaceae family. It comprises approximately 65 genera and 1000 species of annual and perennial herbaceous plants, shrubs and some trees occurring in tropical, subtropical and temperate regions. Previous chemical studies on some of the species reported the occurrence of betacyanines, betaines, ecdysteroids, flavonoids, protoalkaloids, saponins, steroids and triterpenes (Ferreira *et al.,* 2004; Salvador *et al*., 2004; Salvador and Dias, 2004; Brochado *et al*., 2003; Pomilio, *et al*., 1992; Zhou *et al*., 1988). Members of this plant family are also used for the extraction of natural pigments such as betaxanthin, betalains and flavonoids, applied as food colorants and antioxidants (Salvador *et al.,* 2006; Cai *et al*., 2003).

The *Alternanthera* genus includes 80 species, many of which have been traditionally used as analgesic and antimicrobial agents and some *Alternanthera* plants have shown *in vitro* and *in vivo* anti-inflammatory and immunomodulatory activities (Guerra *et al.,* 2003; Moraes *et al.,* 1994). *A. maritima* (Mart.) St. Hil. is a herbaceous plant commonly found on the sandy beaches of the Bra-

zilian eastern coast (Henriques *et al.*, 1984; Siqueira and Guimarães, 1984). The antimicrobial activity of its extracts and isolated flavones was reported by Salvador *et al.* (2003). Chemical analyses have also demonstrated the occurrence of flavonoids, saponins and steroids in *A. maritima* extracts (Salvador *et al.,* 2004; Salvador and Dias, 2004). Preliminary evaluations of *A. maritima* ethanolic extracts revealed high levels of polyphenolic compounds as well as a remarkable antioxidative activity.

The relationship between chemical constitution and biological activities in the plant extracts described above prompted us to investigate the *in vitro* free radical scavenging and immunomodulatory properties of *A. maritima* extracts, fractions and isolated compounds by using chemiluminescence assays. One of the biological activities was evaluated by a cell-free reaction based on the horseradish peroxidase-catalyzed oxidation of luminol in the presence of hydrogen peroxide, and the other involved ROS generation by stimulated human PMNLs. In addition, lactate dehydrogenase (LDH) release determinations and the Trypan Blue exclusion test were used to investigate cytotoxic effects on PMNLs.

Materials and Methods

Chemicals

Horseradish peroxidase type VI-A (HRP, EC 1.11.1.7), luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) and zymosan A were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Dimethylsulfoxide (DMSO; Merck-Schuchardt, Hohenbrunn, Germany), gelatin (Difco Laboratories, Detroit, MN, USA), hydrogen peroxide (H_2O_2; Labsynth, Diadema, SP, Brazil), LDH Liquiform™ (Labtest Diagnostica, Lagoa Santa, MG, Brazil) and Triton X-100 (Union Carbide, Houston, TX, USA) were the other chemicals used in the *in vitro* bioassays. Organic solvents were purchased from Labsynth.

NMR and HPLC analyses

The ^1H, ^{13}C NMR and 2D NMR spectra were recorded in DMSO-d_6 on a Bruker Avance DRX spectrometer operating at 400 and 500 MHz for ^1H, and at 100 and 125 MHz for ^{13}C NMR. ESI-MS was performed using a Micromass Quattro LC system both in the positive (30 V) and negative (30 V) modes. IR spectra were recorded in KBr discs on a Perkin Elmer model 1420 spectrophotometer. UV spectra were recorded on a Hitachi U-3501 spectrophotometer.

Phytochemical studies involved analysis of fractions by reverse-phase high-performance liquid chromatography (HPLC) using a LC-6A Shimadzu liquid chromatograph equipped with a 3501 UV detector operating at 280 nm. Shim-pack ODS (C-18, 4.6×250 mm) and Shim-pack ODS (C-18, 20×250 mm) columns were used for analytical and preparative procedures, respectively. A H_2O/methanol (MeOH) gradient elution profile was applied at room temperature as follows: 30% to 60% MeOH (15 min); 60% MeOH (up to 40 min); 60% to 30% MeOH (5 min); 30% MeOH (5 min); H_2O/MeOH (3:2) was used as the mobile phase in isocratic separations.

Plant material

Aerial parts of *Alternanthera maritima* (Mart.) St. Hil. (Amaranthaceae) were collected at Restinga de Maricá, Rio de Janeiro, RJ, Brazil, in December 1998, and identified by Professor Dr. Josafá Carlos de Siqueira (Pontifícia Universidade Católica, Rio de Janeiro, RJ, Brasil). A voucher specimen was deposited at the Herbarium of the Departamento de Biologia, Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto, Universidade de São Paulo, SP, Brasil (register number SPFR 4758).

Preparation of A. maritima extracts and isolation of compounds

Air-dried, powdered aerial plant parts (5.0 kg) were exhaustively extracted by maceration at room temperature with ethanol (EtOH), and successively filtered and concentrated on a rotatory evaporator (below 40 °C) under reduced pressure, yielding 376 g of ethanolic crude extract. The extract was suspended in MeOH/H_2O (9:1, v/v), partitioned with hexane and dichloromethane and the hydroalcoholic phase was chromatographed on an Amberlite XAD-2 column by sequential elution with H_2O and EtOH. The concentrated ethanolic eluate (120 g) was suspended in MeOH/H_2O (1:4) and partitioned with *n*-butanol (*n*-BuOH) and H_2O resulting in an *n*-BuOH-soluble portion (8 g), which was chromatographed over an 100×5 cm Sephadex LH-20 column and eluted with MeOH (0.5 mL/min). The 110 fractions (10 mL each) collected were monitored by thin layer chromatogra-

phy [TLC, silica gel; *n*-BuOH/acetic acid (AcOH)/H_2O, 65:15:25], grouped in 6 major fractions according to similarity of R_f values and purified by reverse-phase HPLC, precipitation and recrystallization.

The following seven flavonoids were isolated:

Quercetin 3-methyl ether (**1**): m = 25 mg; ESI-MS: m/z = 315 $[M - H]^-$.

Quercetin (**2**): m = 20 mg; ESI-MS: m/z = 301 $[M - H]^-$.

Quercetin 3-O-α-L-rhamnosyl-(1→6)-β-D-glucopyranoside (**3**): m = 3 mg; ESI-MS: m/z = 447 $[M - H]^-$.

Isorhamnetin 3-O-α-L-rhamnosyl-(1→6)-β-D-glucopyranoside (**4**): m = 60 mg; ESI-MS: m/z = 609 $[M - H]^-$, 579 $[M + H]^+$, 633 $[M + Na]^+$.

2″-O-β-D-Glucopyranosyl-vitexin (**5**): m = 25 mg; ESI-MS: m/z = 593 $[M - H]^-$, 595 $[M + H]^+$, 617 $[M + Na]^+$.

2″-O-α-L-Rhamnopyranosyl-vitexin (**6**): m = 140 mg; ESI-MS: m/z = 577 $[M - H]^-$, 579 $[M + H]^+$, 601 $[M + Na]^+$.

Acacetin 8-C-α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranoside (**7**): m = 6 mg; ESI-MS: m/z = 591 $[M - H]^-$, 593 $[M + H]^+$, 615 $[M + Na]^+$.

Isolation of human PMNLs

Blood from healthy human donors was drawn by venous puncture into Alsever solution (v/v) as anticoagulant. PMNLs were isolated by modified previously published methods (Kabeya *et al*., 2002; Lucisano and Mantovani, 1984). Cell pellets suspended in Hank's Balanced Salt Solution (HBSS) containing 0.1% gelatin (w/v) (HBSS-gel) were >90% viable as determined by the Trypan Blue exclusion test and 80–90% were PMNLs. The Local Research Ethics Committee (protocol HCRP 10097/2002) approved the experimental procedure. Opsonized zymosan (OZ) was prepared by the method of Cheung *et al.* (1983) with slight modifications (Kanashiro *et al.,* 2004).

Cellular chemiluminescence assays

The assay was performed as described by Kanashiro *et al.* (2004). Briefly (concentrations of each component in a final reaction volume of 1.0 mL are indicated in parentheses), luminol (160 μmol/L) and aliquots of each sample (6.25–100 μg/mL of ethanolic crude extract or its butanolic fraction; 3.1–50 μmol/L of flavonoids) dissolved in DMSO were added to PMNL suspensions (1×10^6 cells/mL). Controls contained DMSO in place of samples at a content of 0.2% (v/v). After 3 min incubation at 37 °C, ROS generation was triggered by addition of OZ (1 mg/mL). Chemiluminescence (CL^{PMNL}) was measured in a luminometer (AutoLumat LB953, EG&G Berthold, Germany) during 15 min at 37 °C. The area under the CL^{PMNL} versus time curves (AUC) was used to calculate the inhibitory activity for each sample as follows: percentage of inhibition = [100 – (AUC sample/AUC control)] × 100. IC_{50} values (concentrations inhibiting CL^{PMNL} by 50%) were used to compare relative activities of the samples tested.

Cell-free chemiluminescence assay

The assay was performed according to Krol *et al.* (1994) with some modifications. Briefly (concentrations of each component in a final reaction volume of 1.0 mL are indicated in parentheses), aliquots of luminol (160 μmol/L) and each sample (ethanolic crude extract or its butanolic fraction at 6.25–100 μg/mL; flavonoids at 3.1–50 μmol/L) dissolved in DMSO were added to H_2O_2 buffered solution (50 μmol/L). Controls contained DMSO instead of samples in the content 2.0% (v/v). The reaction tubes were incubated for 3 min at 30 °C and ROS generation was initiated by adding HRP (0.2 IU/mL of 0.1 mol/L sodium phosphate buffer, pH 7.4). Chemiluminescence (CL^{HRP}) production was measured in a luminometer for 15 min at 30 °C. CL^{HRP} inhibition was expressed as percentages, as described for the cellular assay. IC_{50} values were used to compare the relative activity of samples.

Cytotoxicity evaluation

The assay was carried out as described by Lucisano-Valim *et al.* (2002). PMNLs (1×10^6 cells/mL) were incubated for 15 min at 37 °C with samples (flavonoids at 50 μmol/L; ethanolic crude extract or its butanolic fraction at 100 μg/mL) or DMSO (control). Total cell lysis (positive control) was achieved with 0.2% (v/v) Triton X-100. Lactate dehydrogenase (LDH) activity released into the supernatant was measured with the test kit LDH Liquiform™. The method is based on decreased NADH absorption at 340 nm measured in a DU-70 spectrophotometer (Beckman, Fullerton, CA, USA) during 3 min at 37 °C. Cell pellets were suspended in HBSS-gel and the cellular viability was

determined by the Trypan Blue exclusion test, based on a count of 200 cells.

Statistical analysis

Analysis of variance (ANOVA) followed by Tukey's test was used to statistically analyze *in vitro* bioassay data. *P*-values < 0.05 were considered statistically significant.

Results

Isolation and identification of compounds in Alternanthera maritima extracts

A. maritima ethanolic crude extracts were partitioned with *n*-BuOH and H_2O and the resulting butanolic fraction was purified, yielding seven compounds (Fig. 1): two flavonoid aglycones (**1** and **2**), two flavonoid *O*-glycosides (**3** and **4**) and three flavonoid *C*-glycosides (**5**, **6** and **7**). Isolated compounds were identified by comparing their physical and spectroscopic properties, including 1D- (^{13}C, DEPT and ^{1}H) and 2D-NMR (TOCSY, HMQC and HMBC), ESI-MS, IR and UV spectra, with those reported in the literature (Salvador *et al.,* 2006; Salvador and Dias, 2004; Aquino *et al*., 2001; Sharaf *et al.*, 1997; Harborne, 1996; Markam and Geiger, 1994; Agrawal, 1989; Matsubara *et al.,* 1984).

Cell-free chemiluminescence inhibition

Free radical scavenging activity of *A. maritima* samples was evaluated by inhibition of chemiluminescence produced by HRP-catalyzed oxidation of luminol (CL^{HRP}) in the presence of H_2O_2. The flavonoid-rich *n*-BuOH fraction was about six times

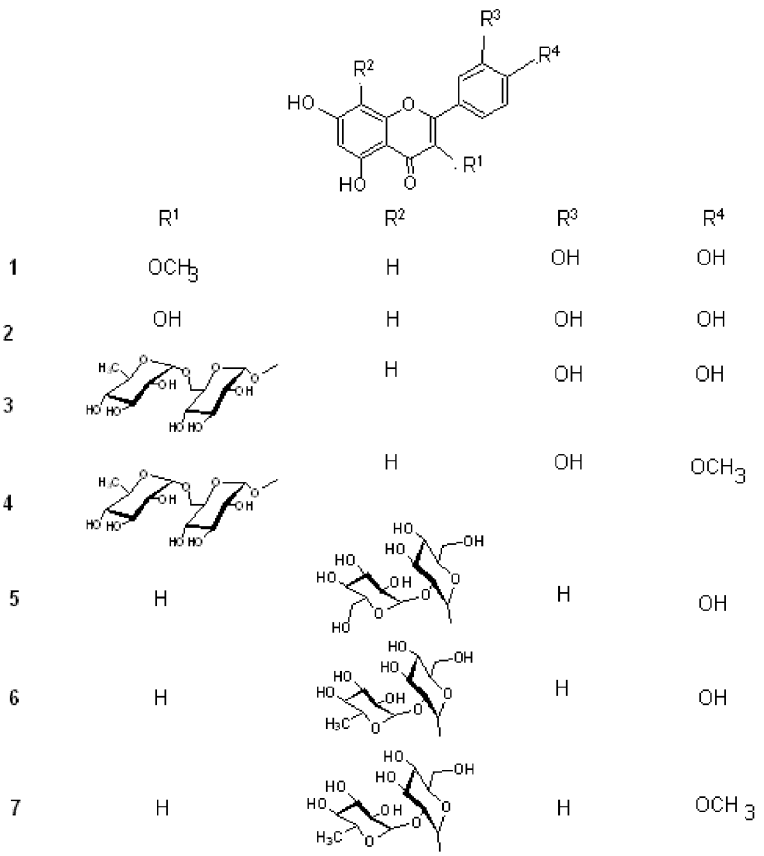

Fig. 1. Structures of compounds isolated from the ethanolic extract of *Alternanthera maritima* aerial parts: quercetin 3-methyl ether (**1**), quercetin (**2**), quercetin 3-*O*-α-L-rhamnosyl-(1→6)-β-D-glucopyranoside (**3**), isorhamnetin 3-*O*-α-L-rhamnosyl-(1→6)-β-D-glucopyranoside (**4**), 2″-*O*-β-D-glucopyranosyl-vitexin (**5**), 2″-*O*-α-L-rhamnopyranosyl-vitexin (**6**) and acacetin 8-*C*-α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranoside (**7**).

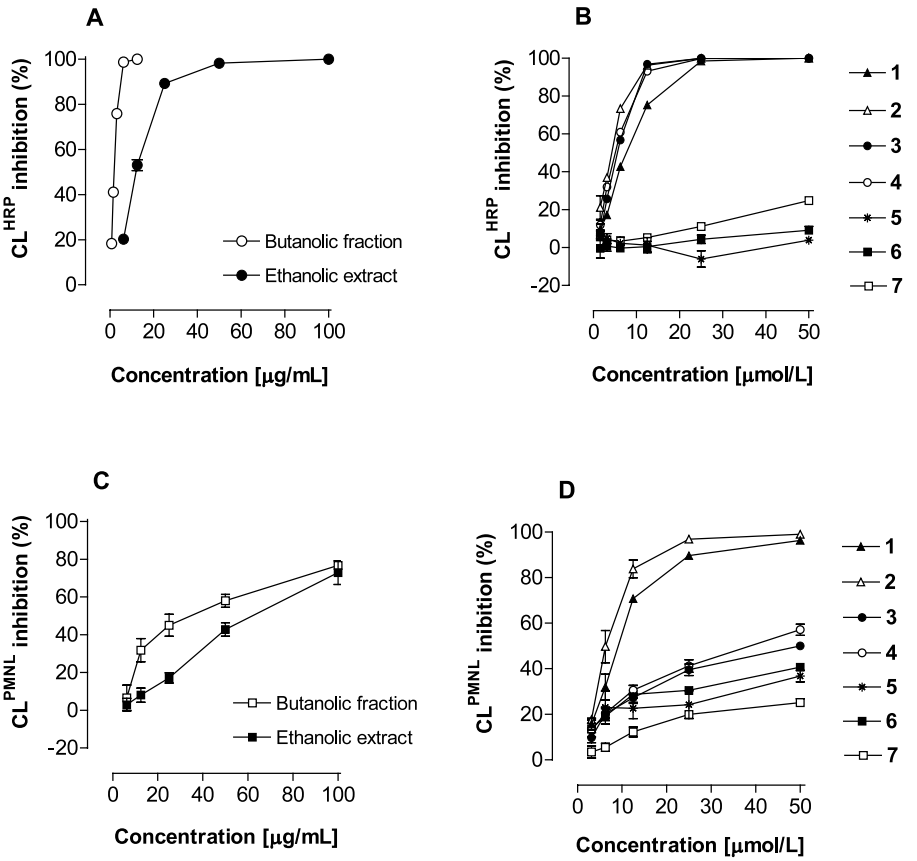

Fig. 2. Effects of *Alternanthera maritima* ethanolic crude extract, *n*-butanolic fraction and isolated compounds in cell-free (A and B) or cellular (C and D) chemiluminescence (CL^{HRP} and CL^{PMNL}, respectively). Data are expressed as percentage of chemiluminescence inhibition *versus* final concentration of the samples in the reaction medium. Compound names and chemical structures are shown in Fig. 1.

more active than the ethanolic crude extract (Fig. 2 and Table I). Flavonoid aglycones **1** and **2** and the *O*-glycosylated compounds **3** and **4** were shown to be significantly more active than the *C*-glycosylated flavonoids **5**, **6** and **7** at the concentrations tested when inhibition percentages and IC_{50} values were compared.

Cellular chemiluminescence inhibition

Immunomodulatory properties of *A. maritima* extracts were assessed by inhibition of OZ-stimulated human PMNL oxidative metabolism, measured by the luminol-enhanced chemiluminescence assay (CL^{PMNL}). The ethanolic crude extract was significantly less active than its butanolic fraction (Fig. 2 and Table I). Among the isolated compounds, flavonoid aglycones **1** and **2** were significantly more active than the *O*-glycosylated compounds **3** and **4**. However, the *C*-glycosylated flavonoids **5**, **6** and **7** had no significant inhibitory activity even at the highest concentration tested (50 μmol/L).

Cytotoxicity evaluation

The cytotoxic effect of experimental samples on PMNLs was investigated, since this could be one of the mechanisms underlying inhibition of cellular chemiluminescence production. The ethanolic crude extract and its butanolic fraction, both tested at 100 μg/mL, and the seven isolated flavonoids at 50 μmol/L did not induce a significant release of LDH when compared to the control (DMSO) (Table II). In addition, the percentage of dead cells, determined by the Trypan Blue exclu-

Sample	CL^{PMNL} IC_{50} †	CL^{HRP} IC_{50} ‡
Ethanolic crude extract [μg/mL]	57.09 ± 4.27$^{\beta}$	11.90 ± 0.44$^{\beta}$
Butanolic fraction [μg/mL]	30.45 ± 5.42$^{\gamma}$	1.88 ± 0.05$^{\gamma}$
Isolated flavonoids [μmol/L]		
1	8.72 ± 0.78^{a}	7.31 ± 0.11^{a}
2	6.32 ± 0.79^{a}	4.17 ± 0.13^{b}
3	43.41 ± 1.46^{b}	5.22 ± 0.07^{c}
4	37.41 ± 1.96^{b}	4.82 ± 0.12^{c}
5	>50	>50
6	>50	>50
7	>50	>50

Table I. Inhibitory effects of *Alternanthera maritima* ethanolic crude extract, its butanolic fraction and isolated flavonoids in the luminol-enhanced chemiluminescence produced by cellular (CL^{PMNL}) and cell-free systems (CL^{HRP}).

Data are expressed as means ± SEM (standard error of the mean). Compound names and chemical structures are shown in Fig. 1.

† CL^{HRP}, chemiluminescence was produced by the luminol-horseradish peroxidase-H_2O_2 reaction ($n = 3$).

‡ CL^{PMNL}, chemiluminescence was produced by opsonized zymosan-stimulated human neutrophils ($n = 4–6$).

IC_{50}, concentration that inhibits chemiluminescence by 50%.

Statistics: ($\beta \neq \gamma$) or (a ≠ b ≠ c ≠ d), $P < 0.001$ (ANOVA and Tukey's *post-hoc* test).

Sample	Cellular viability † (%)	LDH activity ‡ (IU × 1000)
Triton X-100	–	117.60 ± 6.02
DMSO	91.50 ± 3.39	6.49 ± 1.11
Ethanolic crude extract [100 μg/mL]	78.50 ± 1.50	1.39 ± 0.07
Butanolic fraction [100 μg/mL]	82.00 ± 1.00	0.76 ± 0.17
Isolated flavonoids [50 μmol/L]		
1	97.75 ± 0.35	5.22 ± 0.27
2	91.25 ± 1.77	6.29 ± 3.20
3	94.50 ± 0.71	5.63 ± 1.01
4	87.00 ± 2.83	4.13 ± 1.46
5	91.50 ± 3.54	4.08 ±1.93
6	87.50 ± 0.71	5.81 ± 1.64
7	93.50 ± 1.41	5.74 ± 2.27

Table II. Cytotoxicity of *Alternanthera maritima* ethanolic crude extract, its butanolic fraction and isolated flavonoids on human PMNLs.

Data are expressed as means ± SEM ($n = 4$). Compound names and structures are shown in Fig. 1.

† Evaluated by Trypan Blue exclusion, based on 200 cell counts.

‡ Activity of lactate dehydrogenase (LDH) released into the supernatant after 15 min incubation with the samples at 37 °C. Triton X-100 (0.2%, v/v) promoted total lysis of 1×10^6 PMNLs, and was used as the positive control.

sion test, was around 10–15% for the control and all samples tested. Together, these results indicate that the ethanolic crude extract, its butanolic fraction or the isolated compounds were not toxic to human PMNLs under these conditions.

Discussion

During the inflammatory process, recruited PMNLs release a variety of ROS that are involved in tissue damage (Babior, 2000). Thus, controlling oxidative metabolism by NADPH-oxidase inhibition and ROS scavenger drugs might be of therapeutic value in the treatment of inflammatory and autoimmune diseases. ROS generation by OZ-stimulated PMNLs, a parameter used to evaluate the immunomodulatory properties of *A. maritima* aerial parts crude extracts and isolated compounds, was inhibited by most samples in a concentration-dependent manner.

As previously reported, PMNL oxidative metabolism inhibition may be due to three central mechanisms: cell death, ROS scavenging and inhibition of signal transduction pathways involved in ROS generation (Van Dyke and Castranova, 1987). No significant viability decrease was found when PMNLs were incubated with the plant extracts or the isolated compounds possibly excluding a cytotoxic effect. However, a further explanation of the mechanism(s) of action is limited by the complexicity of the cellular system. The observed effects might be the result of multiple interactions between enzymatic targets such as NADPH-oxidase (Tauber *et al.*, 1984), myeloperoxidase (MPO), phosphoinositide 3-kinase (Shelloum *et al.*, 2001), phospholipase D and MAP-kinase (Takemura *et al.,* 1997).

On the other hand, it is known that flavonoids scavenging activity is generally related to the pres-

ence, number and position of hydroxy, methoxy and glycoside groups in their chemical structure. A decisive role is played by a hydroxy group situated at C-3 in the C ring with an adjacent C-2–C-3 double bound (Kanashiro *et al.,* 2004; Rice-Evans *et al.,* 1996). In this study, substitution of the hydroxy group in position C-3 (compound **2**) by a methoxy group reduced the inhibitory effect of compound **1** in the cell-free system. The presence of glycoside groups or a cathecol group in the B ring are other important features of the antioxidative activity of flavonoids (Rice-Evans *et al.,* 1996; Odontuya *et al.,* 2005). In fact, either a B ring cathecol group (R^3 and R^4 dihydroxy groups in the B ring) or glycoside groups in the C ring influence the ability to reduce the generation of ROS. In the present study, the activity of aglycone compounds **1** and **2** and *O*-glycosides **3** and **4** was higher than of *C*-glycosides **5**, **6** and **7** in a cell-free system.

However, when compared to *O*-glycosides, aglycone compounds were more effective in the cellular systems. Compounds having glycoside residues in the A ring C-8 position but lacking a hydroxy or methoxy group at C-3 position in the B ring showed a low inhibitory activity in both systems. The observed activity difference between aglycones and glycosylated flavonoids may be explained, in part, by their physicochemical properties including appropriate lipophilicity (Rothwell *et al.,* 2005), which characterizes molecules able to cross the cellular membrane. Generally, because glycosides are hydrophilic they are unavailable to the cell interior and thus, not able to interact with specific targets such as intracellular enzymes and ROS. Accordingly, *in vivo* studies have demonstrated that glycosylated flavonoids are not found inside intestinal epithelial cells (Day *et al.,* 2003) or blood plasma (Morand *et al.,* 2000) because they are converted to their free aglycone form by the intestinal microflora for posterior absorption (Walle, 2004). On the other hand, a recent study has reported that the presence of a glucose group, but not rhamnose, improves the absorption of quercetin in the intestine by a glucose carrier-dependent mechanism (Morand *et al.*, 2000). This study suggests that the presence or position of specific glycoside groups can increase or decrease the bioavailability of flavonoids. In addition, many studies have proposed the use of glycoside groups containing compounds in conjunction with drugs as a new therapeutic strategy to control their absorption and bioavailability (Walle, 2004).

In summary, the present work showed that *A. maritima* may be considered a promising natural source of antioxidant compounds for therapeutic or preventive uses against ROS-dependent chronic diseases. Further studies investigating the role of bioactive extracts and flavonoids from *Alternanthera* species in other biological systems are necessary to better define their therapeutic potential.

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